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L6: Entry 3 of 8 File: USPT May 15, 2001

DOCUMENT-IDENTIFIER: US 6232075 B1
TITLE: Heterogeneous assay for pyrophosphate detection

Abstract Text (1):
Nucleotide triphosphate probes containing a fluorophore attached to the .beta.-phosphate and a quencher moiety sufficiently proximal to the fluorophore moiety for use in pyrophosphate detection assays are disclosed. These probes exhibit distinguishable fluorescence characteristics when the fluorophore is attached to the nucleotide through the .gamma.-phosphate and when it is unattached to the nucleotide. The present invention also provides kits and integrated systems for practicing the assays described herein.

Brief Summary Text (2):
This invention relates generally to a heterogeneous assay, and in particular, to assay methods using fluorescent nucleotide triphosphates having a fluorophore moiety attached to the .gamma.-phosphate that are especially useful for pyrophosphate detection.

Brief Summary Text (7):
Fluorophore-quencher pairs have been incorporated into oligonucleotide probes in order to monitor biological events based on the fluorophore and quencher being separated or brought within a minimum quenching distance of each other. For example, probes have been developed wherein the intensity of the fluorescence increases due to the separation of the fluorophore-quencher pair. Probes have also been developed which lose their fluorescence because the quencher is brought into proximity with the fluorophore. These fluorophore-quencher pairs have been used to monitor hybridization assays and nucleic acid amplification reactions, especially polymerase chain reactions (PCR), by monitoring either the appearance or disappearance of the fluorescence signal generated by the fluorophore molecule.

Brief Summary Text (12):
Nucleotide triphosphates having a fluorophore moiety attached to the .gamma.-phosphate are of interest as this modification still allows the modified NTPs to be enzyme substrates. For instance, Felicia et al., describe the synthesis and spectral properties of a "always-on" fluorescent ATP analog, adenosine-5'-triphosphoro-.gamma.-1-(5-sulfonic acid)-naphthyl ethylamindate (.gamma.-1,5-EDANS)ATP. The analog is a good substrate for E. Coli RNA polymerase and can be used to initiate the RNA chain. The ATP analog is incorporated into the RNA synthesized and is a good probe for studies of nucleotide-protein interactions, active site mapping and other ATP-utilizing biological systems (see, Felicia et al., Arch. Biochem Biophys., 246: 564-571 (1986)).

Brief Summary Text (14):
A need currently exists for effective nucleotide triphosphate molecules containing a fluorophore and a quencher for use in pyrophosphate detection assays. Accordingly, a need exists for assays using probes which exhibit distinguishable fluorescence characteristics when a fluorophore is attached to the nucleotide through the .gamma.-phosphate and when it is unattached to the nucleotide. A further need exists for assays using probes wherein the fluorophore and a quencher are positioned on the probe such that the quencher moiety can effectively quench the fluorescence of the fluorophore moiety. These and further objectives are provided by the methods and probes of the present invention.

Brief Summary Text (16):
A need currently exists for effective nucleotide triphosphate molecules containing a fluorophore and a quencher for use in pyrophosphate detection assays. Pyrophosphate

detection is useful for monitoring a number of enzymatic reaction mechanisms such as nucleic acid polymerase reactions. As such, in certain aspects, the present invention provides a heterogeneous assay method for detecting pyrophosphate cleavage, the components of the assay comprising a labeled NTP, a target nucleic acid, a primer nucleic acid and a polymerase, the method comprising:

Brief Summary Text (17):
(a) flowing the labeled nucleotide triphosphate (NTP) having a .gamma.-phosphate with a fluorophore moiety attached thereto and a quencher moiety sufficiently proximal to the fluorophore moiety to prevent fluorescence of the fluorophore moiety, past an immobilized component selected from the group consisting of the polymerase and the target nucleic acid;

Brief Summary Text (20):
Preferably, in the methods of the present invention, the enzyme is immobilized on a solid support and the nucleotide triphosphates comprise dATP, dCTP, dGTP, dTTP, dUTP, ATP, CTP, GTP, UTP and mixtures thereof. The detection of the fluorescent moieties is preferably accomplished using single molecule detection with for example, a charge couple device (CCD) camera.

Brief Summary Text (21):
In another aspect, the present invention provides a nucleotide triphosphate (NTP) probe, comprising: a NTP having a .gamma.-phosphate with a fluorophore moiety attached thereto; a quencher moiety sufficiently proximal to the fluorophore moiety to prevent fluorescence of the fluorophore moiety; wherein the fluorophore moiety exists quenched with at least about a 5 fold quenching efficiency when the .gamma.-phosphate is attached to the NTP and unquenched when the .gamma.-phosphate is detached from the NTP. In preferred aspects, the quencher moiety is attached to the nucleobase.

Detailed Description Text (5):
The term "nucleotide" as used herein refers to a phosphate ester of a nucleoside, e.g., mono, di and triphosphate esters, wherein the most common site of esterification is the hydroxyl group attached to the C-5 position of the pentose. Nucleosides also include, but are not limited to, synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described generally by Scheit, Nucleotide Analogs (John Wiley, N.Y., 1980). Suitable NTPs include both naturally occurring and synthetic nucleotide triphosphates, and are not limited to, ATP, dATP, CTP, dCTP, GTP, dGTP, TTP, dTTP, UTP and dUTP. Preferably, the nucleotide triphosphates used in the methods of the present invention are selected from the group of dATP, dCTP, dGTP, dTTP, dUTP and mixtures thereof.

Detailed Description Text (13):
In certain embodiments, the present invention provides a heterogeneous assay for the detection of pyrophosphate. The detection of pyrophosphate is advantageous in a number of biological reactions. For example, in a DNA polymerase reaction, wherein the polymerase selects a single DNA molecule from solution and thereafter incorporates the nucleotide at the 3'-end of a primer strand, the natural consequence of such incorporation is the release of pyrophosphate. If the assay solution comprises the four deoxynucleotide triphosphates, each dNTP labeled with a different color of fluorescent dye attached to the .gamma.-phosphate, it is then possible to sequentially record the activity of the polymerase operating on a target DNA. The nucleotide sequence of the target DNA can thereafter be read directly from the order of released dyes attached to the pyrophosphate.

Detailed Description Text (14):
As such, the present invention provides a heterogeneous assay method for detecting pyrophosphate cleavage, the components of the assay comprising a labeled NTP, a target nucleic acid, a primer nucleic acid and a polymerase, the method comprising: (a) flowing the labeled nucleotide triphosphate (NTP) having a .gamma.-phosphate with a fluorophore moiety attached thereto and a quencher moiety sufficiently proximal to the fluorophore moiety to prevent fluorescence of the fluorophore moiety, past an immobilized component selected from the group consisting of the polymerase and the target nucleic acid; (b) incorporating the NTP on a primer strand hybridized to the target nucleic acid using an enzyme and releasing the .gamma.-phosphate with the fluorophore moiety attached thereto; and (c) detecting the fluorescent moiety thereby

detecting pyrophosphate cleavage. In the heterogeneous assay of the present invention, either the polymerase or the target nucleic acid is attached to a solid phase, such as a solid support. Preferably, in the methods of the present invention, the polymerase is immobilized on a solid support.

Detailed Description Text (16):
In certain aspects of the present invention, a labeled nucleotide triphosphate (NTP) having a .gamma.-phosphate with a fluorophore moiety attached thereto is incorporated into a polynucleotide chain. As illustrated in FIG. 1A, dNTP incorporation into a growing oligonucleotide by a DNA polymerase results in pyrophosphate cleavage. In this reaction, the phosphate ester bond between the .alpha. and .beta. phosphates of the incorporated nucleotide is cleaved by the DNA polymerase, and the .beta.-.gamma.-diphosphate (pyrophosphate) is released in solution. As used herein, the term pyrophosphate also includes substitution of any of the oxygen atoms of the pyrophosphate group with a nitrogen or a sulfur atom or combinations thereof to generate thiopyrophosphate, dithiopyrophosphate, etc.

Detailed Description Text (17):
As shown in FIG. 1B, in compounds of the present invention wherein a fluorophore is attached to the .gamma.-phosphate, the fluorophore is released from the nucleotide along with the pyrophosphate group. In certain aspects, cleavage of the pyrophosphate switches the fluorophore moiety into a fluorescent state i.e., the fluorophore is dequenched. This event can then be detected using an ultrasensitive fluorescence detector. Using single molecule detection for example, fluorescent signals appear at the locations of the individual molecules being observed. In certain aspects, each type of nucleotide is labeled with a different fluorophore so that the incorporated nucleobases can be sequentially identified by the released fluorophores. Preferably, the nucleotide triphosphate (NTP) of the present methods include, but are not limited to, deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate, deoxyuridine triphosphate or mixtures thereof, each with a unique fluorophore attached to the .gamma.-phosphate.

Detailed Description Text (19):
Single molecule detection using methods of the present invention is illustrated in FIG. 2. In certain embodiments, an unlabeled, single-stranded target nucleic acid with a primer hybridized thereto is tethered to the surface of a solid support such as a glass slide. An aqueous solution comprising an enzyme, such as a DNA polymerase, and fluorogenic dNTPs flows across the surface. Alternatively, in another embodiment, an individual polymerase molecule is immobilized on a glass slide and the polymerase is bathed in a flowing solution comprising: 1) unlabeled, single-stranded DNA fragments hybridized to an oligonucleotide primer and 2) a mixture of deoxynucleotide triphosphates, each uniquely labeled with a different color of fluorescent dye attached to the .gamma.-phosphate.

Detailed Description Text (31):
The target nucleic acid can be prepared by various conventional methods. For example, target nucleic acid can be prepared as inserts of any of the conventional cloning vectors, including those used in conventional DNA sequencing. Extensive guidance for selecting and using appropriate cloning vectors is found in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references. Sambrook et al. and Innis et al, editors, PCR Protocols (Academic Press, New York, 1990) also provide guidance for using polymerase chain reactions to prepare target polynucleotides. Cloned or PCR-amplified target nucleic acid is prepared which permit attachment to solid supports.

Detailed Description Text (45):
In certain aspects, the methods of the present invention comprise detecting and identifying individual fluorogenic dNTP molecules as a polymerase incorporates them into a single DNA molecule. In certain aspects, a fluorescent dye is attached to the .gamma.-phosphate and a quencher is attached to the nucleobase. As such, the present invention provides a nucleotide triphosphate (NTP) probe, comprising: a NTP having a .gamma.-phosphate with a fluorophore moiety attached thereto; a quencher moiety sufficiently proximal to the fluorophore moiety to prevent fluorescence of the fluorophore moiety; wherein the fluorophore moiety exists quenched with at least about a 5 fold quenching efficiency, preferably, at least a 10 fold quenching efficiency,

when the .gamma.-phosphate is attached to the NTP and unquenched when the .gamma.-phosphate is detached from the NTP. In preferred aspect, the NTP probe is a dNTP probe having a fluorescent dye attached to the .gamma.-phosphate moiety and a quencher attached to the nucleobase. Suitable nucleobases include, but are not limited to, adenine, guanine, cytosine, uracil, thymine, deazaadenine and deazaguanosine. The quenched dNTPs are non-fluorescent when the .gamma.-phosphate is attached to the NTP, and thereafter become fluorescent when the .gamma.-phosphate is unattached to the NTP.

Detailed Description Text (49):
In another embodiment, the fluorophore and quencher function by an electron transfer mechanism. In this aspect, a non-fluorescent quencher e.g. DABCYL or dinitrophenyl (see, FIG. 3) absorbs energy from an excited fluorophore, but does not release the energy radiatively. These quenchers can be referred to as chromogenic molecules.

Detailed Description Text (102):
The quenching efficiency of DABCYL-dUTP-BODIPY TR was determined as follows. First, the fluorescence of a sample containing the dye BODIPY TR is measured. Second, a sample containing the same concentration of the nucleotide triphosphate having a .gamma.-phosphate with a fluorophore moiety attached i.e., DABCYL-dUTP-BODIPY TR is measured. Thereafter, the quenching efficiency, which is equal to F.sub.o -F/ F.sub.o wherein F.sub.o is fluorescence of the BODIPY TR alone and F is the fluorescence of DABCYL-dUTP-BODIPY TR is calculated. The fluorescence quenching efficiency of DABCYL-dUTP-BODIPY TR is at least 5 fold compared to the BODIPY TR alone.

Other Reference Publication (45):
Schecker et al., "Flow-based continuous DNA sequencing via single molecule detection of enzymatically cleaved fluorescent nucleotides", Proc. SPIE--Int. Soc. Opt. Eng. 2386:4-12 (1995).

Other Reference Publication (48):
Smagowicz et al., "Properties of P.sup.3 Esters of Nucleoside Triphosphates as Substrates for RNA Polymerase from Escherichia coli," Biochem., 20:5538-5546 (1981).

Other Reference Publication (56):
Wu et al., "Synthesis and Properties of Adenosine-5'-triphosphoro-.gamma.-1-(5-sulfonic acid)naphthyl Ethylamidate: A Fluorescent Nucleotide Substrate for DNA-Dependent RNA Polymerase from Escherichia coli," Arch. Biochem. Biophys., 246:564-567 (1986).

Other Reference Publication (59):
Yarbrough, et al., "Synthesis and properties of fluorescent nucleotide substrates for DNA-dependent RNA polymerases," JBC, 254:12069-12073 (1979).

CLAIMS:

1. A heterogeneous assay method for detecting pyrophosphate cleavage, the components of the assay comprising a labeled NTP, a target nucleic acid, a primer nucleic acid and a polymerase, said method comprising:

(a) flowing said labeled nucleotide triphosphate (NTP) consisting of a .gamma.-phosphate with a fluorophore moiety attached thereto and a quencher moiety sufficiently proximal to said fluorophore moiety to prevent fluorescence of said fluorophore moiety past an immobilized component selected from the group consisting of said polymerase and said target nucleic acid;

(b) incorporating said NTP on a primer strand hybridized to said target nucleic acid using said polymerase and releasing said .gamma.-phosphate with said fluorophore moiety attached thereto; and

(c) detecting said fluorescent moiety thereby detecting pyrophosphate cleavage.

2. The method according to claim 1, wherein said nucleotide triphosphate (NTP) is a member selected from the group consisting of deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxyguanosine triphosphate and deoxythymidine

triphosphate.

3. The method according to claim 1, wherein said nucleotide triphosphate (NTP) is a member selected from the group consisting of adenosine triphosphate, cytosine triphosphate, guanosine triphosphate and uridine triphosphate.

6. The method according to claim 1, wherein said nucleotide triphosphate (NTP) is a plurality of nucleotide triphosphates (NTPs).

7. The method according to claim 1, wherein each of said plurality of nucleotide triphosphates (NTPs) has an indicator of identity.

12. A nucleotide triphosphate (NTP) probe, said NTP probe consisting of:

a NTP having a .gamma.-phosphate with a fluorophore moiety attached thereto;

a quencher moiety sufficiently proximal to said fluorophore moiety to prevent fluorescence of said fluorophore moiety;

wherein said fluorophore moiety exists quenched with at least about a 5 fold quenching efficiency when said .gamma.-phosphate is attached to said NTP and unquenched when said .gamma.-phosphate is detached from said NTP.

14. The NTP probe according to claim 13, wherein said NTP is a member selected from the group consisting of a deoxynucleotide triphosphate (dNTP), and a nucleotide triphosphate (NTP).

15. The NTP probe according to claim 14, wherein said NTP is a deoxynucleotide triphosphate (dNTP).

16. The NTP probe according to claim 15, wherein said deoxynucleotide triphosphate (dNTP) is a member selected from the group consisting of deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate.

17. The NTP probe according to claim 15, wherein said nucleotide triphosphate (NTP) is a member selected from the group consisting of adenosine triphosphate, cytosine triphosphate, guanosine triphosphate and uridine triphosphate.

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